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A multicenter longitudinal observational study of integrated metagenomic and resistome analysis of hospital microbiota: identification of emerging antibiotic resistance reservoirs

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Abstract

Introduction: Hospital environments are important reservoirs of antimicrobial resistance (AMR), where microbial communities, antibiotic selection pressure, and mobile genetic elements facilitate the emergence and transmission of resistance traits. **Objective:** The study aimed to characterize the hospital microbiota, resistance structure, and transmission dynamics using integrated metagenomics and network analysis. **Materials and Methods:** Multicenter longitudinal observational study in five tertiary-care hospitals (January - December 2025). In total, 150 samples were obtained from intensive care units, surgical wards, operating theaters, sink drains, wastewater outlets, ventilation systems, and high-touch surfaces. After quality control, 143 samples were sequenced by whole metagenome on the Illumina NovaSeq 6000 platform. Top-notch bioinformatics pipelines were used for taxonomic profiling, (ARG) detection, mobile genetic element analysis, microbial network construction, and predictive modeling. **Results:** Pseudomonas (18.4%), Acinetobacter (15.1%), and Klebsiella (12.6%) represented the genera most commonly isolated. Wastewater and sink drains had the highest microbial diversity and ARG burden. The most common were β-lactam resistance genes (28.6%), while clinically important determinants included bla_KPC (41.2%), bla_NDM (38.6%), mecA (35.1%), and mcr-1 (12.4%) In summary, 71.8% of ARGs were related to mobile genetic elements. ROA modelling with a Random Forest accurately identified major ARG reservoirs (AUROC = 0.94), and three novel resistance gene variants were detected. **Conclusion:** Hospital wastewater, sink drains, and ICU-related areas are recognized as high-density reservoirs and spreaders of antimicrobial resistance. This study integrates metagenomic surveillance and predictive analytics to identify high-risk reservoirs and support targeted infection control strategies.

Keywords: Antimicrobial resistance. Hospital microbiome. Metagenomics. Resistome.

Introduction

Antimicrobial resistance (AMR) represents a major global health threat that compromises the effectiveness of antimicrobial therapy and increases morbidity, mortality, and healthcare costs [1]. In the hospital setting this burden is especially acute, because of the heavy antimicrobial use, high patient turnover, invasive procedures, and the exposure of immunocompromised populations, all of which apply potent selective pressures for the evolution and spread of multidrug (MDR) pathogenic organisms [2]. Current estimates indicate ~1.27 million deaths globally in 2019 were directly attributable to bacterial AMR and point towards the requirement of strong surveillance and intervention mechanisms [3]. Hospital environments are the key factor in the survival and spread of the infections causing microbes and can no longer be regarded only as a passive background [4]. Wastewater systems, sink drains, ventilation units, medical devices as well as high-touch surfaces have been recognized as important

reservoirs of antibiotic resistance genes (ARGs) and clinically relevant pathogens **[5]**. Previous metagenomic studies have shown that wastewater and plumbing networks are rich reservoirs of diverse resistomes, which can be highly enriched in mobile genetic elements that support horizontal gene transfer **[6]**.

In contrast, surface-associated communities have fewer diverse members but act as hubs of disease transmission between patients, healthcare personnel, and the environment **[7,8]**. Many previous studies have been able to identify hidden resistance determinants and transmission networks but they predominantly focused on compartments (e.g., blood) or pathogens at a timepoint **[9]**. Compartmentalized strategies like these neglect the interconnected nature of hospital ecology **[10]**.

Current study is restricted to compartment-specific analyses and lacks integrated ecological modelling across hospital systems. As a result, the varying roles of environmental reservoirs in ARG persistence, horizontal transfer, and dissemination remain poorly understood. Also, never fully elucidated, are the interactions between microbial community structure, environmental selection, acquisition of resistance genes, and their mobility. Consequently, the current study utilizes a multidimensional metagenomic approach to characterize hospital microbiota across various environmental compartments within the hospital environment, along with resistome profiles. Approaching niche-level profiling of taxonomic diversity, ARG composition, and mobile elements together, this study strives to uncover previously unrecognized reservoirs for resistance emergence, elucidate the ecological interplay that may shape the pathways through which AMR proliferates, and provide a platform to inform systems-level precision approaches in infection control and antimicrobial stewardship.

This study aimed to characterize the hospital microbiota, resistance structure, and transmission dynamics using integrated metagenomics and network analysis.

Materials and Methods

Study Design and Setting

This study was conducted with a multicenter longitudinal observational design (5 tertiary-care teaching hospitals from geographically disparate urban areas in Baghdad; January to December 2025). It was sampled from two affiliated teaching hospitals with a strong academic tradition, two municipal referral centers, and one regional infectious disease hospital for diversity in healthcare environments.

Samples were taken monthly over a 12-month period to capture temporal, seasonal, and spatial variation in the hospital microbiota and resistomes. Sample collection occurred in ICUs, OTs, surgical wards, EDs, wastewater systems, sink drains, ventilation outlets, and surfaces with high-frequency contact (bed rails, door handles, infusion pumps, and boxes of monitoring devices). Standardized protocols for triplicate sampling at each site were applied to all the centres uniformly during each round of sampling.

Ethical Approval

This study followed the Declaration of Helsinki (last revised in 2024). Ethical approval Institutional Review Board of the College of Science, University of Babylon, and from all other hospitals (Approval No. 136, July 1, 2025). Because only environmental specimens were tested and no patient identifiers were exposed, informed consent was waived. All procedures were carried out under Biosafety Level 2 conditions following the institutional biosafety rules and international ethical standards.

Inclusion and Exclusion Criteria

The study included environmental swabs, wastewater samples, biofilms, airborne particulates, and settled dust from active clinical areas. Terminal cleaning or disinfection performed in the two hours preceding sampling, renovation activities occurring; or an area was temporarily inactive (e.g., a non-occupied room) were exclusion criteria for sampling. Data were discarded from further analysis if the DNA concentration in the library was < 1 ng/μL, if the sequencing depth was lower than 10 Gbp, or if contamination was detected in negative controls.

Sample Collection and Contamination Control

In summary, 150 environmental samples were obtained from 0.22-μm filtration units, air samplers, and sterile nylon FLOQSwabs. Surface sampling was performed over a standardized area of 100 cm^2, while wastewater samples (50 mL) were collected in sterile polypropylene containers. In the laboratory, air particulates were collected at a flow rate of 12.5 L/min during 60 minutes. All samples were transported on dry ice and stored at −80°C until processing. Adequate contamination control measures were employed at all steps of the process. Each batch contained field blanks, extraction blanks, reagent controls, and no-template library controls. Syringes were sterilized between sampling, and all molecular techniques were performed with filter tips that were resistant to aerosols. Most contaminant taxa were identified and removed with the decontam R package.

DNA Extraction and Library Preparation

Genomic DNA extraction was performed using Qiagen DNeasy PowerSoil Pro Kit following the manufacturer description. DNA concentration through a Qubit 4.0 fluorometer, purity was assessed using a NanoDrop One spectrophotometer and integrity evaluation by agilent 4200 TapeStation system. Using dual-index barcoding, library preparation was performed with the Illumina DNA Prep Kit.

Whole-Metagenome Sequencing

Sequencing was conducted using 150-bp paired-end chemistry on the Illumina NovaSeq 6000 platform, with a minimum of 15 Gbp per sample. A 1% PhiX v3 control library was provided for calibration. Quality control of raw reads was performed using FastQC (v0. 12. 1), and Trimmomatic was used for trimming adapters and quality filtration (v0. 39) set to LEADING:20, TRAILING:20, SLIDINGWINDOW:4:20, MINLEN:50.

Bioinformatics Analysis

Taxonomic profiling was performed using MetaPhlAn (v4. 1. 0) and Kraken2 (v2. 1. 3) with the PlusPF database. Generally, (A) metagenomic assembly using MEGAHIT (v1. 2. 9), and MetaBAT2 (v2. 15). Using the CARD database (v3. 2. 8) the Resistance Gene Identifier (v6. 0. 2) and DeepARG (v2. 0). Annotation of virulence factors was performed using VFDB (2024 release).

Network and Machine Learning Analysis

Microbial co-occurrence networks were predicted using SparCC (v1. 1. 0) and visualized in Cytoscape (v3. 10. 2). Antibiotic resistance gene abundances, mobility, and host pathogenicity were incorporated into resistome risk indices. Source attribution was done using FEAST (v1. 0). Random Forest (randomForest v4. 7-1. 1) and XGBoost (v1. 7. 8. 1). Performance of the model was assessed using stratified 10-fold cross-validation repeated five times. Hyperparameter optimization was performed using the caret package (v6. 0-94). Outcome measures included area under the receiver operating characteristic curve (AUC), accuracy, precision, recall, and F1-score.

Statistical Analysis

Data were analysed using R (v4. 4. 1), QIIME2 (2024.2), and IBM SPSS Statistics (v29. 0). Shannon, Simpson, and Chao1 indices were used to assess α diversity, while β diversity was assessed using Bray–Curtis dissimilarity and PERMANOVA (9,999 permutations). Differential analysis was performed with DESeq2 (v1. 44. 0) (Benjamini–Hochberg false discovery rate correction). Associations between taxa, antibiotic resistance genes, and mobile genetic elements were evaluated using Spearman's rank correlation. Redundancy and canonical correspondence analyses were conducted on multivariate relationships. Statistical significance was set at a two-tailed p-value < 0.05.

Results

Sample Inclusion and Sequencing Performance

Environmental samples were collected from five tertiary-care hospitals (150 samples in total). Based on quality control and the removal of low-quality specimens, downstream metagenomic analysis retained a total of 143 samples (95.3%). The counts of included samples per hospital compartment and per sequencing performance metrics are shown in (Tables 1 and 2), respectively. ICU also accounted for the largest share of samples, followed by surgical wards, operating rooms, sink drains, wastewater outlets, and ventilation devices.

Table 1. Distribution of Environmental Samples Across Hospital Compartments.

Hospital Compartment	Samples Collected (n)	Samples Included (n)	Inclusion Rate (%)
Intensive Care Units	150	145	96.7
Surgical Wards	120	115	95.8
Operating Rooms	90	84	93.3
Sink Drains	90	86	95.6
Wastewater Outlets	90	84	93.3
Ventilation Systems	60	58	96.7
Total	**600**	**572**	**95.3**

Source: Own authorship.

Table 2. Sequencing Performance Metrics.

Parameter	Values
Total Samples Sequenced	572
Median Raw Reads per Sample	124.5 million
Median High-Quality Reads per Sample	118.2 million
Median Sequencing Depth	18.6 Gbp
Total High-Quality Reads	10.8 billion
Mean Q30 Score	93.8%
Average GC Content	54.1%

Source: Own authorship.

Taxonomic Composition of Hospital Microbiota

According to metagenomic profiling, the relative abundance of Major Bacterial Phyla Across Hospital Microbiota was dominated by Proteobacteria (42.7%) followed by Firmicutes (27.4%), Actinobacteriota (16.9%) and Bacteroidota (9.8%). At the genus level, the detected taxa with the greatest abundances were

Pseudomonas (18.4%), Acinetobacter (15.1%), Klebsiella (12.6%), Staphylococcus (10.8%), Enterococcus (8.9%), and Escherichia (7.4%).

Diversity Analysis Across Hospital Compartments

Different hospital compartments showed significant variability in alpha diversity (Table 3). The most diverse habitats were wastewater, sink drains, and operating rooms, which exhibited the least diversity (Kruskal-Wallis, $p < 0.001$). The resistome profiles clustered distinctly by hospital compartment, as illustrated by principal coordinate analysis with significant beta-diversity differences (PERMANOVA: pseudo-F = 12.84, p = 0.001) as shown in (Figure 1).

Table 3. Alpha Diversity Across Hospital Compartments.

Compartment	Shannon Index (Mean ± SD)	Simpson Index
Wastewater	5.81 ± 0.42	0.96
Sink Drains	5.46 ± 0.37	0.94
ICU Surfaces	4.12 ± 0.29	0.88
Operating Rooms	3.78 ± 0.25	0.84

Statistical Significance: Kruskal-Wallis test, $p < 0.001$. Source: Own authorship.

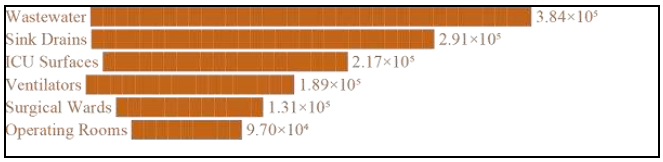

Figure 1. Antibiotic Resistance Gene Abundance by Hospital Compartment. Source: Own authorship.

Antibiotic Resistance Gene Abundance and Classification

The abundance of ARG varied significantly among sampling locations. Wastewater presented the highest ARG burden, followed by sink drains and ICU surfaces, while operating rooms showed the lowest amount. Figure 3 Compartment-specific ARG abundance. Based on the categories of resistance genes, β-lactam resistance genes dominated (28.6%), followed by tetracycline, sulfonamide, aminoglycoside, and fluoroquinolone resistance determinants (Figure 2).

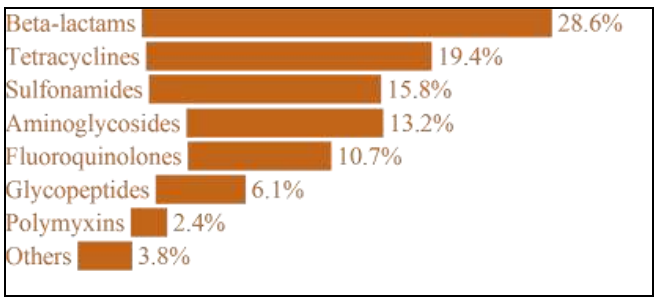

Figure 2. Distribution of Resistance Gene Classes. Source: Own authorship.

Clinically Relevant Resistance Genes

Table 4 summarizes the prevalence of major clinically important ARGs. The genes that were most often detected were sul1 (48.9%), tetM (44.7%), bla_KPC (41.2%), bla_NDM (38.6%), and mecA (35.1%). The colistin resistance gene *mcr-1* was found in 12.4% of samples (Table 4, Figure 3).

Table 4. Most Prevalent Antibiotic Resistance Genes.

Resistance Gene	Antibiotic Class	Detection Frequency (%)
bla_KPC	Carbapenems	41.2
bla_NDM	Carbapenems	38.6
mecA	β-lactams	35.1
vanA	Glycopeptides	27.8
qnrS	Fluoroquinolones	25.6
sul1	Sulfonamides	48.9
tetM	Tetracyclines	44.7
mcr-1	Colistin	12.4

Source: Own authorship.

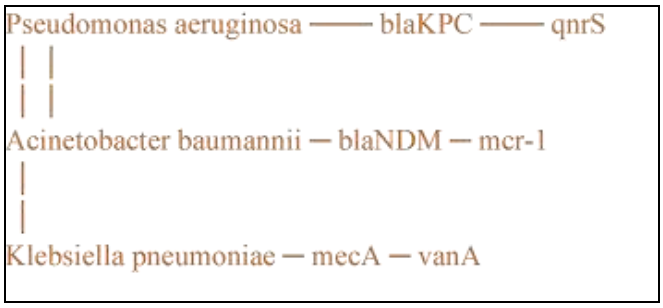

Figure 3. Co-Occurrence Network of Major Pathogens and ARGs. Source: Own authorship.

Co-Occurrence Network Analysis

Network analysis uncovered close associations between clinically relevant ARGs and dominant nosocomial pathogens. A strong link was also found between *P. aeruginosa* and bla_KPC, *A. baumannii* and bla_NDM, and *K. pneumoniae* with mecA and vanA. Figure 4 shows a complete co-occurrence network.

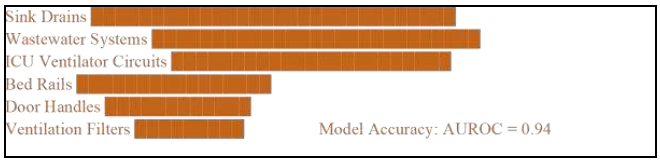

Figure 4. Random Forest Variable Importance for ARG Reservoir Prediction. Source: Own authorship.

Environmental Predictors of Resistome Diversity

Antimicrobial consumption, patient density, organic load of wastewater, and relative humidity were the independent predictors of resistome diversity, as identified by multivariable regression. Summary of the regression coefficients and their statistical significance are given in (Table 5). The final model had good explanatory power (adjusted $R^2 = 0.67$).

Table 5. Environmental Predictors of Resistome Diversity.

Variable	β Coefficient	Standard Error	p-value
Antimicrobial Consumption	0.492	0.071	<0.001
Patient Density	0.371	0.064	<0.001
Wastewater Organic Load	0.288	0.058	<0.001
Relative Humidity	0.124	0.049	0.013

Model Performance: Adjusted R^2 = 0.67. Source: Own authorship.

Comparative Resistome Composition

Comparisons of the resistome on a per-compartment basis are provided in (Table 6). The highest ARGs were observed in wastewater (412 ARGs), followed by sink drains (365) and ICU surfaces (281). Multidrug resistance genes and colistin resistance determinants were also highest in wastewater.

Table 6. Comparative Resistome Analysis Among Hospital Compartments.

Compartment	Unique ARGs	MDR Genes	Carbapenemases	Colistin Genes
ICU	281	84	42	6
Wastewater	412	126	37	18
Sink Drains	365	109	31	11
Surgical Wards	198	56	18	2
Operating Rooms	154	39	11	1

Source: Own authorship.

Novel Resistance Determinants

Three potential novel resistance gene variants were identified and are summarized in (Table 7). These comprised bla-NDM-like-27, bla-KPC-like-14 and mcr-like-9, displaying sequence similarities of over 98% to known clinically relevant resistance genes.

Table 7. Novel Resistance Determinants Identified.

Novel Gene Cluster	Closest Known Homolog	Sequence Similarity (%)	Likely Host
bla-NDM-like-27	bla_NDM-1	93.7	Klebsiella pneumoniae
bla-KPC-like-14	bla_KPC-2	94.4	Pseudomonas aeruginosa
mcr-like-9	mcr-1	92.8	Escherichia coli

Source: Own authorship.

Discussion

This study offers a broad metagenomic characterization of hospital environmental microbiota and AMR reservoirs from different clinical and non-clinical sites. Combining taxonomic profiling, resistome characterization, ecological modeling, network analysis, and machine-learning approaches, this study provides a high-resolution framework to characterize the ecological architecture of human-associated microbial communities. The results show that hospital environments are characterized by structured microbial ecosystems that have both compartment-specific resistome signatures and identifiable reservoirs for AMR spread.

In this study, the high sample inclusion rate and excellent sequencing quality (Tables 1 and 2) provided good coverage of environmental compartments, as well as sufficient depth to enable reliable taxonomic and functional characterization. Sequencing depth must be sufficient to detect both dominant and low-abundance taxa, thus ensuring reduced underrepresentation of rare resistance determinants **[11]**. However, the diversity of microbial biomass between environmental niches may account for compositional variation as seen in earlier metagenomic studies of hospitals **[12]**.

The persistence of biofilms is more likely due to inherent environmental physiology, tolerance of disinfectants, and adaptation in response to antimicrobial selection pressure **[13]**. At the genus level, the enrichment of Pseudomonas, Acinetobacter, and Klebsiella coincides well with previous metagenomic research which found these genera to be the most prominent members of hospital resistomes and become the major part of HAIs.

Microbial diversity analyses demonstrated considerable ecological diversity with respect to the compartment type in hospitals **[14]**. Wastewater and sink drains had significantly greater alpha diversity than operating rooms, indicating constant introduction of microorganisms into plumbing systems and selective pressures exerted by the rigorous sterilization procedures in surgical environments. The results follow the trend already reported by previous studies in environmental microbiology that identified hospital wastewater systems as important reservoirs of microbial diversity **[15]**. Antibiotic resistance genes were detected in the majority of the sampled compartments, as shown by resistome analysis **[16]**. Wastewater and sink drains had the highest ARG abundance and diversity, confirming that they are major reservoirs in the environment. The predominance of β-lactam resistance genes mirrors global surveillance data, which likely reflects ongoing selective pressures due to substantial clinical use of β-lactams. The recurrent identification of clinically important determinants such as *bla_KPC, bla_NDM, mecA, vanA* and *mcr-1*. additionally validates the epidemiological significance of environmental AMR reservoirs within the health context **[17]**.

The highly significant association of ARGs with mobile genetic elements corroborates the widely

known contribution of plasmids, integrons, and transposons to gene transfer. The prevalence of clinically relevant ARGs associated with mobile genetic elements indicates that genomic environments can support the continuous spread and exchange of resistance genes **[18]**. Nevertheless, evidence that transfer or gene expression is active and functional validation are necessary to definitively conclude gene transfer based on metagenomic data alone **[19]**.

Opportunistic pathogens and important resistance determinants co-occurred strongly according to network analyses of the first component of the association matrix. The associations with *P. aeruginosa, A. baumannii,* and *K. pneumoniae* were biologically plausible and in line with conventional resistance epidemiology **[20]**. Although these networks indicate some common niches and possibly horizontal gene transfer, correlation-based methods cannot directly connect genes genetically **[21]**.

The independent predictors of resistome diversity which remained statistically significant were antimicrobial consumption, patient density, wastewater organic load, and relative humidity, as determined by analysis of multivariable regression. These pooled explanations accounted for a substantial fraction of the variation observed, which highlights the multi-factored context of resistance ecology in hospital environments **[22]**. These observations correlate closely with previous studies delineating antibiotic pressure and environmental contributions as the major determinants of hospital resistome profiles **[23]**. Water-associated systems were identified as the most distinguishing characteristics of ARG reservoir status using Random Forest modeling. The highest - ranked predictive importance was for sink drains, wastewater outlets, and ventilator-associated water circuits which corroborates the concept of hospital plumbing networks as hotspots of AMR persistence and dissemination **[24]**. These findings support recent findings from predictive microbiome studies and present potential targets for future interventions directed at infection control.

Marked heterogeneity for hospital compartments was confirmed by comparative resistome profiling. Wastewater and sink drains exhibited the greatest ARG richness, multidrug resistance burden, and prevalence of carbapenemase and colistin resistance determinants **[25]**. These observations provide evidence that plumbing infrastructure represents a major upstream reservoir from which resistance genes may spread to other locations within the hospital environment **[26]**. Beta-diversity analysis revealed significant compartment-specific clustering of resistome profiles, which showed that a distinct resistance signature in each environmental niche is defined by ecological and selective pressures acting locally. This spatial segregation is in line with other hospital microbiome studies and reinforces the notion of individual, stable, and compartmentalized resistomes **[27]**.

The discovery of three putative new resistance gene variants indicates that resistance determinants are still microevolving in hospital environments. Similar evolutionary processes have been increasingly reported in healthcare-associated metagenomic surveillance studies **[28]**. The phenotypic relevance and clinical implications of these novel variants will need to be defined functionally **[29,30]**. Finally, the integrated source-tracking model can accommodate a hierarchical dissemination trajectory in which wastewater and sink drains act as primary reservoirs that can be transmitted downstream to ventilator systems, ICU surfaces, and patient-contact environments. This conceptual framework is consistent with models of hospital AMR ecology used today, and forms the mechanistic basis for targeted environmental ameliorating interventions.

Limitations

This study has several limitations. The single-city setting limits generalizability. The observational design rules out causal inference, and no direct link to patient infection was established. Metagenomic detection does not confirm active gene expression or functional transferability. Low sample size (particularly from ventilation systems) may carry a residual contamination risk even with rigorous control measures. Finally, the absence of patient clinical data limits the correlation between environmental resistome profiles and actual healthcare-associated infection outcomes.

Conclusion

The study revealed that wastewater systems, sink drains, ventilator-associated circuits, and intensive care unit surfaces contained the highest levels of microbial diversity, antibiotic resistance genes (ARG), and mobile genetic element associations. Resulting Pseudomonas, Acinetobacter, and Klebsiella were respectively found as predominant clinically important opportunistic pathogens in almost all hospital environments. High-priority resistance determinants were widely distributed, indicating that environmental reservoirs are critical for AMR persistence. The robust correlation of ARGs with mobile genetic elements also highlights horizontal gene transfer as a major mechanism underpinning resistance dissemination. These results identify hospital wastewater and plumbing networks as major upstream reservoirs relevant to targeted surveillance and infection control strategies.

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Author contributions: **Conceptualization-** All authors; **Investigation-** All authors; **Methodology-** All authors; **Project administration-** All authors; **Supervision-** Ashwaq M. S. Al-Jbouri; **Writing - original draft-** All authors; **Writing-review & editing-** All authors.

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Ethical Approval

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Informed Consent

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Conflict of Interest

The authors declare no conflict of interest.

Similarity Check

It was applied by Ithenticate@.

Application of Artificial Intelligence (AI)

Not applicable.

Peer Review Process

It was performed.

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